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HEMOGLOBIN-SPECTRIN COMPLEXES: INTERFERENCE WITH SPECTRIN TETRAMER ASSEMBLY AS A MECHANISM FOR COMPARTMENTALIZATION OF BAND 1 AND BAND 2 COMPLEXES

BY

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Abstract

The irreducible complexation of hemoglobin with spectrin is a natural phenomenon of red cell aging, positively correlating with increasing cell density and decreasing cell deformability. The current study begins to address the role of these complexes in the disruption of membrane skeletal physiology and structure. The effect of bound hemoglobin on spectrin dimer self-association was investigated in vitro. The extent of conversion of isolated spectrin dimers to tetramers was evaluated as a function of peroxide-induced globin complexation prior to the conversion incubations. The incremental accumulation of tetramer was observed to decrease with increasing peroxide concentration used in the globin complexation step. The role of oxidized heme in this process was made apparent by the inability of carboxyhemoglobin to inhibit tetramer accumulation. A Western blot analysis of naturally formed globin-spectrin conjugates demonstrated irreducible complexes of globin with both bands 1 and 2. The complexes are tentatively designated "h1" and "h2". This analysis also demonstrated that h1 is completely extractable from cell ghosts, whereas h2 is only 50% extractable. These findings are incorporated into a hypothesis linking globin-spectrin complexation and the consequent inhibition of spectrin dimer self-association to the clustered band 3 senescence antigen (Low et al. (1985) *Science* 227, 531-533).

Introduction

The decline of red cell deformability with cell age is a function of changes in cell shape, cytoplasmic viscosity, and membrane deformability. Membrane deformability, in turn, is crucially dependent on the ability of the cytoskeletal spectrin network to undergo unfolding and refolding. When circulation stresses result in forces beyond the limit of reversible deformability, then cytoskeletal protein junctional complexes are temporarily broken. Among the weakest of these junctions are the head-to-head associations of the spectrin dimers.[1]

We had previously shown that irreducible complexation of hemoglobin with spectrin is a natural phenomenon of red cell aging, positively correlating with increasing cell density and decreasing cell deformability.[2-4] Experimentally, in vitro exposure of peroxide results in signs of accelerated cell senescence, including generation of hemoglobin-spectrin complexes, decreased cell deformability, and cell surface alterations that lead to enhancement of phagocytosis by monocytes.[5,6] Significantly, prior treatment of human red cells with carbon monoxide completely inhibits the cellular alterations induced by peroxide, a finding which is consistent with the established role of hemoglobin oxidation as the initial step in the production of oxygen free radicals in red cells.[7-9]

The present study takes a more direct approach to the question of what effect

bound hemoglobin has on spectrin dimer self-association. In other words, does the interposition of hemoglobin into a spectrin dimer-dimer junction under conditions of membrane shear stress constitute the wedge which initiates diminishing membrane deformability? Ektacytometric measurements of elliptocytic red cell membranes have shown that the increased spectrin dimer:tetramer ratio in these membranes was associated with a decrease in deformability to one-tenth the normal value.[10] The stability of these membranes was reduced as well, but only to 0.26-0.28 the normal level. Thus, uncoupled spectrin dimers appear to affect membrane deformability more adversely than they affect membrane stability. Moreover, the potential exists that globin-bound uncoupled dimers may facilitate an increased lateral mobility of attached band 3 molecules, and thus their clustering into the senescence antigens described by Low et al.[11]

Spectrin extracted from membranes at 37°C is almost entirely in the form of $\alpha\beta$ heterodimers which easily undergo reversible self-association in dilute solution at 30°C to form $\alpha_2\beta_2$ tetramers.[12,13] At 4°C, there is little or no interconversion. Our experimental design is to test whether peroxide-induced binding of hemoglobin to spectrin dimers results in a diminished capability of spectrin self-association into tetramers, beyond that due to peroxidation of spectrin alone. We also demonstrate by Western blotting the existence of two types of natural globin-spectrin complexes within the membrane skeleton and show a partitioning of these two types within the skeleton on the basis of their differential extractability.

Materials and Methods

Preparation of red blood cell ghosts and spectrin extracts. Venous human blood was drawn on heparin and immediately processed. Red cells were washed (x3) in 5 mM sodium phosphate buffer (pH 7.4)/150 mM NaCl. Membrane ghosts were prepared by hypotonic lysis in 5 mM sodium phosphate (pH 8.0)/1 mM EDTA, in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (0.1 mM) at 40 volumes lysis buffer per volume packed cells. Alternatively, ghosts were prepared from packed cells by freeze/thaw lysis (dry ice/methanol bath). Residual hemoglobin in the ghosts was removed by incubating for 10 min at 0°C with lysis buffer in the presence of 0.5 mM diisopropyl fluorophosphate (DFP), followed by a final wash in 0.1 mM sodium phosphate (pH 8.0)/0.1 mM EDTA/0.1 mM DTE.

For the preparation of spectrin tetramer extracts, ghosts were incubated for 16 h at 0°C with 10 mM sodium phosphate (pH 8.0) using one volume of extraction buffer per volume packed cells. Extracted ghosts were then pelleted by centrifugation at 243,000 x g for 1 h at 4°C, and the spectrin extract in the supernatant was purified by sucrose density gradient centrifugation (below).

Spectrin extracts were also prepared from red cells which had been treated with hydrogen peroxide, as previously outlined.[5] Briefly, washed red cells were adjusted to a hematocrit of 20% in glucose-enriched phosphate-buffered saline with 1.0

mM NaN_3. Hydrogen peroxide was added to the cell suspension to final concentrations of 0, 349 or 849 μM. The cell suspension with peroxide was then incubated for 15 min in a shaking water bath. Ghosts and spectrin extracts were then prepared as described above.

Spectrin purification. Sucrose step gradients were used for isolation of spectrin tetramers from ghost extracts (above) and for isolation of peroxide-generated hemoglobin-spectrin complexes (below) from free hemoglobin. Step gradients were prepared in 12.7 x 50.8 mm ultracentrifuge tubes by sequential overlay of 0.9 ml each of 20% (w/v) sucrose with 17%, 13%, 9% and 5% sucrose in 10 mM Tris-HCl (pH 8.0). Spectrin extracts (above), up to 0.5 ml, were overlaid onto the step gradients and centrifuged at 82,500 x g, 4°C, for 16 to 18 h. Fractions of 0.2 to 0.3 ml were sequentially removed from the top of the gradient. Protein concentration of each fraction was determined from absorption at 280 nm, using $E^{1\%}_{1cm}$ human erythrocyte spectrin = 8.8.[14]

Preparation of hemoglobin for complexation with spectrin. Venous human blood was drawn on heparin, red cells were pelleted and washed (x3) in 10 mM sodium phosphate (pH 7.4)/150 mM NaCl. Packed cells were vortexed with an equal volume of distilled water and subjected to three freeze/thaw cycles (dry ice/methanol bath). Cell membranes were pelleted at 48,000 x g, 4°C, 30 min, and the hemolysate supernatant was recovered by aspiration. Hemoglobin A was

isolated from the hemolysate by anion-exchange chromatography using DE52 cellulose (Whatman, Inc., Clifton, NJ), batch-eluting with 5 mM sodium phosphate (pH 7.4). Protein concentration was determined photometrically (S PLUS, Coulter, Miami, FL).

Preparation of globin-complexed soluble spectrin. Hemoglobin A was complexed in vitro with soluble spectrin by the heme-catalyzed Fenton reaction.[15] Reactions of HbA (6 μM, 0.39 mg/ml) with spectrin (2.4 μM, 1.25 mg/ml) were initiated with H_2O_2 (147 or 489 μM) in 10 mM sodium phosphate (pH 7.4)/150 mM NaCl, incubating 15 min at 37°C. Control incubations of spectrin included: 1) no HbA, no H_2O_2; 2) HbA, no H_2O_2; 3) no HbA, 147 μM H_2O_2; 4) no HbA, 489 μM H_2O_2; and 5) HbA preincubated with CO for 10 min at 20°C, 489 μM H_2O_2. The HbA-spectrin reactions were then adjusted to 20 mM DTE/0.5 mM DFP/0.1 mM EDTA, incubated a further 15 min at 37°C, followed by dialysis against 10 mM sodium phosphate (pH 8.0) at 4°C. The spectrin preparations were then isolated from free HbA by sucrose step gradients as described above.

Analysis of soluble spectrin self-association. (1) Dimer-tetramer interconversion. Following the isolation of globin-spectrin complexes from free HbA, the spectrin dimers were allowed to reversibly convert to tetramers. These reactions were carried out at 1.25 mg/ml in 5 mM sodium phosphate (pH 7.4)/150 mM NaCl/1 mM DFP/0.1 mM DTE, and allowed to reach equilibrium by incubation for 4 h at

30°C.[12] The uncomplexed spectrin controls were incubated similarly.

(2) Agarose-acrylamide gel electrophoresis and calculation of dimer-tetramer conversion. The spectrin dimer-tetramer mixtures were evaluated both before and after the 4 hour interconversion incubations, using electrophoresis under non-denaturing conditions on 0.3% agarose, 2.5% acrylamide composite cylindrical gels run at 4°C.[16] The gels were stained with Coomassie Brilliant Blue and then scanned (model 1038 densitometer, Helena Laboratories, Beaumont, TX). From the scan printout, the peak areas corresponding to the dimer and tetramer bands were carefully cut out and weighed to the nearest 0.05 mg. The extent of conversion from dimer to tetramer forms during the four hour incubation was expressed as the percentage of spectrin in the tetrameric form, ie, tetramer weight/(tetramer + dimer weight) at 4 hours less that at 0 hours. The percent conversion at each time point for each trial mixture was averaged from two runs.

Calculation of extractable spectrin. Ghosts and spectrin extracts from peroxide-treated cells (described above) were electrophoresed on 4% SDS-PAGE (Fairbanks buffer system)[17] cylindrical gels, then stained and scanned as described for spectrin dimer-tetramer analysis. The unextracted ghosts used for SDS-PAGE analysis were suspended in the same volume as that used for the spectrin extractions. From the scan printouts, the peak areas corresponding to the spectrin bands were cut out and weighed. Extracted spectrin was expressed as percent

total spectrin from the corresponding ghost preparation. The percent extracted spectrin was averaged from three runs.

Quantitation of globin-spectrin complex concentration. (1) Western blots of cell ghosts, extracted spectrin, and residual membranes. SDS-PAGE of cell ghosts, extracted spectrin and residual membranes employed the Laemmli buffer system,[18] a 6% separating gel in the mini format (55x85x0.75 mm) (Bio-Rad Laboratories), and a 5-slot, 4% stacking gel. One lane was reserved for high molecular weight markers (45-200 kDa, Bio-Rad Laboratories) to calculate M_r values of reactive sample bands. Samples of known protein content (bicinchoninic acid method; Pierce, Rockford, IL) were heated (100°C, 5 min) in sample buffer containing dithioerythreitol and electrophoresed (200V) so that the pyronin Y marker dye traversed 5.0 cm. The electrophoresed samples were transferred (150 mA) to a nitrocellulose membrane (0.10 μm pore, Micron Separations Inc., Honeyoye Falls, NY). After transfer, lane positions were determined and sample lanes were split vertically. For each sample lane, one of the half-lanes was stained for total protein using colloidal gold (Bio-Rad Laboratories). The other half-lane was probed with the anti-globin monoclonal antibody (hh-1-294), 15 μg IgG/ml, 1 h, 25°C. The buffer TTBS (0.2% Tween 20 in TBS: 20 mM Tris-HCl, pH 7.50, 500 mM NaCl) was used to block the membrane prior to antibody incubation, to carry both first and enzyme-conjugated second antibodies, and to wash the membrane after each antibody incubation. Development of the blot was then carried out with

bromochloroiodophenyl/nitroblue tetrazolium (Moss Inc., Pasadena, MD), 15 min, 25°C.

(2) Densitometry. Densitometric analysis of the developed blots was carried out at 540 nm reflectance, scanning each half-lane (7-8 mm width) three times at 2 mm intervals with a 0.05 x 2.0 mm beam, using a CS-9000 scanner and the CSTURBO 1D Analysis Program (Shimadzu Corp., Kyoto, Japan). Antibody-reactive bands identified by molecular weight shifts as globin-spectrin complexes were densitometrically integrated by the analysis program, converting average band colorimetric intensities into area units. Quantities of each globin-spectrin complex were expressed both as raw area units and as specific activities relative to total sample protein. The gold-stained components in the residual membrane sample were similarly scanned and integrated. The gold-stained globin complex of band 2 ("h2") was incompletely resolved from band 2 on the chromatogram, resulting in an integrated area encompassing both the band 2 peak and the preceeding h2 shoulder. A conservative estimate of the h2 area was made by extrapolating the leading edge of the band 2 peak back to the baseline. The divided shoulder and peak were then cut from the chromatogram and weighed to three significant figures. The weights were then converted to area units based on the original combined peak integration.

Preparation of anti-globin monoclonal antibody. The monoclonal antibody used in

the Western blots (hh-1-294; mouse IgG1,κ) recognizes both beta and alpha human hemoglobin subunits in the blot format. The hybridoma secreting this antibody was the fusion product of the mouse myeloma cell line FOX-NY and immune splenic B lymphocytes derived from an RBF/Dn mouse immunized with human HbA. The methodology for immunization, for production, screening, cloning and characterization of the hybridoma/monoclonal antibody, and for production and isolation of IgG from ascites fluid essentially followed that previously described for the human HbA (β6 Glu specific) antibody.[19]

The reactivity of hh-1-294 vs isolated globin chains was assessed by dot blot. Alpha and beta globins were isolated from hemoglobin by ion exchange chromatography with urea buffers,[20] applied (1 μg) to nitrocellulose, probed with the monoclonal IgG (15 μg/ml), and developed as described for Western blots (above).

Results

Effect of globin complexation on soluble spectrin self-association. The potential of bound hemoglobin to obstruct spectrin dimer self-association was tested in a cell-free system, measuring shifts in dimer-tetramer ratios at equilibrium as a function of peroxide concentration used in the globin complexation step. The results of the soluble spectrin dimer-tetramer equilibration trials are presented in Fig. 1. Each

stained gel pair depicts dimer and tetramer concentrations both at the start and after the four hour equilibration. The incremental accumulation of tetramer is seen to be much less in the case of spectrin complexed with globin at 489 μM H_2O_2 (Fig. 1F, 4 h vs 0 h) than occurs with spectrin treated with the same concentration of peroxide alone (Fig. 1E) or with the untreated spectrin (Fig. 1A). Spectrin complexed with globin at 147 μM H_2O_2 shows a less inhibited accumulation of tetramer (Fig. 1D vs 1C or 1A). It is noted that the concentration of soluble tetramer at the start of the incubation appears to be higher for the globin complexed spectrins than for the corresponding peroxide-treated or untreated spectrins (Fig. 1F, 0 h vs 1E or 1A, 0 h; Fig. 1D, 0 h vs 1C or 1A, 0 h). Even the treatment by hemoglobin without peroxide appears to induce a small increase in tetramer concentration at 0 h (Fig. 1B). As the reaction conditions prior to the start of the incubation (hypotonic environment, 0°C) do not favor tetramer formation,[21] we suggest that the apparent increase in tetramer concentration at 0 h, particularly with hemoglobin in the oxidized state, reflects an intermolecular cross-linking of spectrin rather than tetramer formation. Supporting this view, carboxyhemoglobin/peroxide-treated spectrin showed no increased tetramer concentration at 0 h (Fig. 1G vs 1E or 1A). Moreover, Western blot analysis of spectrin + hemoglobin ± peroxide, incubated as described for globin complexation (Methods) and then brought to hypotonic, 0°C conditions, revealed an irreducible high MW band which reacted with monoclonal antibodies specific for hemoglobin, spectrin α and spectrin β (unpublished data). Although we have not yet definitively

established the relative molecular mass of this band, we tentatively conclude that this represents globin-complexed cross-linked spectrin heterodimers.

To compensate for elevated pre-equilibration tetramer backgrounds, cited above, in assessing the self-association trials, the parameter used to compare the variously treated spectrins was the incremental increase in tetramer concentration expressed as a percentage difference, ie, $T_4/(T_4+D_4)-T_0/(T_0+D_0)$ where T and D refer to the tetramer and dimer integrated concentrations, respectively, at 4 or 0 hours of incubation. The incremental percent tetramer increases for control spectrin, globin-spectrin complexes at 147 and 489 μM H_2O_2, and carboxyhemoglobin/peroxide treated spectrin are presented in Table 1, which quantifies the equilibration trials illustrated in Fig. 1A, 1D, 1F and 1G. It is noted that the incremental increase in tetramer accumulation decreases with increasing concentration of H_2O_2 used to create the oxyhemoglobin-complexed spectrin.

Spectrin extractability. To determine the effect on self-associational properties of spectrin extracted from peroxide-treated red cells, similar dimer-tetramer equilibration studies as described above were carried out. While there was no observable effect on self-association of spectrin extracted from treated cells (data not shown), it was observed that the extractability of spectrin was inversely proportional to the concentration of peroxide used. Table 2 summarizes the results of the spectrin extractability studies on peroxide-treated cells. While treatment with

349 μM H_2O_2 did not appreciably affect spectrin extractability, treatment with 849 μM H_2O_2 rendered spectrin about three times less extractable than in untreated cells — 58% inextractable at 849 μM vs 20% inextractable at 0 μM H_2O_2.

Immunoquantitation of complexes in cell ghosts, extracted spectrin and residual membranes. Identification and quantitation of globin-spectrin complexes in cell ghosts before and after extraction of spectrin was carried out by densitometric analysis of Western blots using an anti-globin monoclonal antibody (hh-1-294).

Figure 2 is a Western blot of untreated cell ghosts before and after extraction, and the extracted spectrin. The hh-1 antibody is seen to react with two high molecular weight bands in the unextracted ghosts and extracted spectrin, and only one high molecular weight band in the extracted (residual) membranes. The split-lane blot clearly shows that the immunoreactive globin complexes are upwardly shifted from the corresponding protein-stained spectrin bands. The relative positions of the complexes are consistent with 1:1 molar ratios of the component M_r values (band 1 = 240 kDa, band 2 = 220 kDa, globin = 16 kDa). The complexes are tentatively designated "h1" and "h2", signifying the irreducible linkages of (hemo)globin with bands 1 and 2, respectively. All of the h1 and a portion of the h2 complexes were coextracted with spectrin. The balance of the h2 complexes remained associated with the residual membranes.

The quantitation of the complexes in Fig. 2 is expressed in terms of densitometric area units, which are also normalized to total protein loaded for electrophoresis (Table 3). The h1 complexes were undetected in the residual membranes, whereas the h2 complexes were about half extracted (46.9 vs 89.3 area units). Correspondingly, the specific activity of both complexes (area units/μg protein) was approximately doubled in the extracted spectrin, whereas the specific activity of h2 was diminished by about one-fourth in the residual membranes. Table 4 lists the relative proportions of the inextractable spectrin (band 1, h2, band 2) as scanned from the gold-stained residual membrane profile in Fig. 2. It can be seen that h2 accounts for about one-third (6.29/19.60 area units) of the total inextractable β spectrin (h2 + band 2).

Discussion

Physiological effects of complexes. Judged by their molecular weight shifts (Fig. 2), the irreducible covalent complexes of globin and spectrin chains occur with apparent equimolar stoichiometry. This suggests that the inhibition of spectrin dimer self-association following globin complexation in vitro (Fig. 1 and Table 1) may result from steric or allosteric effects of globin binding at or near the head region. At this point, our data on the specifics of globin-spectrin binding is limited to the identity of the globin chains composing the h1 and h2 complexes from this study and our previous report.[4] Previously, we had shown that a β globin-specific

monoclonal antibody (β^{10}-1-18946), recognizing an epitope centered approximately at β10 Ala, detected an h1 complex found in sickle cell membranes, but no h2 complex. As the monoclonal antibody used in the current study (hh-1-294) recognizes both β and α globin, one may conclude that the h2 complex contains α globin. Thus, the human h1 complex is β globin-α spectrin, and the h2 complex is α globin-β spectrin. Preliminary data from the baboon system indicates that the globin compositions are reversed, ie, α globin-α spectrin and β globin-β spectrin (not shown).

An alternative interpretation of the inhibited dimer self-association following globin complexation may be that the junctional sites on the spectrin subunits are oxidatively damaged by hydroxyl radicals generated from H_2O_2 by the heme iron in the complexed globin, as described below. In a cellular context, this would probably require that the location of the complexed hemoglobin be at or near the dimer-dimer junction because of the free radical's limited effective range.[22]

Lamchiagdhase et al[23] have reported defective spectrin dimer self-association in thalassemic red cells. In their study, spectrin was extracted from various hemoglobinopathic subjects and dimer-tetramer conversion measurements were carried out by methods similar to those described herein. Spectrin extracted from severely thalassemic cells showed significantly defective dimer self-association properties, with β-thalassemic cells (excess, unpaired α globin) being more

defective than α thalassemic.

Compartmentalization of complexes. The h2 globin-spectrin complexes are far less extractable than the h1, remaining in the residual membranes (Fig. 2 and Table 3). This suggests that h2 complexes (but not h1) are tightly associated with integral membrane proteins, eg, the anion channel band 3 protein, through atypical interactions catalyzed or mediated through the globin components. The h2 complex (human α globin-β spectrin) appears to be associated with disrupted membrane skeletal structure, which can be appreciated in β-thalassemic cells. Yuan et al[24] have demonstrated that the erythroid precursors of severely β-thalassemic cells exhibit accumulative α globin deposition beginning as early as the proerythroblast stage. Preliminary double-immunolabeling studies[25] have demonstrated sites of colocalization of α globin with spectrin in these precursors. At these sites, the spectrin often appeared clumped rather than exhibiting its usual smooth distribution (rim fluorescence).

Although we have used peroxide to experimentally accelerate the rate of formation of globin-spectrin complexes, the complexes themselves did not appear to differ physiologically from those encountered naturally (as shown in Fig. 2). Thus, peroxide-generated h1 complexes were completely extractable regardless of the peroxide concentration used (not shown). We suggest that under natural conditions of oxidative stress, the heme iron of the spectrin-bound globin catalyzes

intermolecular covalent bonding through generation of hydroxyl radicals from H_2O_2 — a natural byproduct of aerobic metabolism. Heme iron has been demonstrated to be capable of catalyzing cyclical generation of hydroxyl radicals via the Fenton reaction.[15] We have demonstrated the natural occurrence of globin-spectrin irreducible covalent bonds (Fig. 2), but do not rule out the possibility that other types of intermolecular crosslinkages may result from the suggested mechanism — cyclical generation of hydroxyl radicals, or from other undefined mechanisms. As pointed out, inextractable h2 accounts for about one-half of total h2 naturally found in unfractioned red cells (from Table 3), but only about one-third of total inextractable β spectrin, ie, h2/(h2+band 2) (from Table 4).

The poor extractability of the h2 complexes is postulated to result from their inclusions in localized disruptions of membrane skeletal structure. A current working hypothesis regarding the formation of the membrane signal for senescent red cell destruction holds that hemoglobin denaturation leads to hemichrome formation, the binding of hemichrome tetramers to high affinity sites on band 3 dimers,[26] and the subsequent clustering of band 3 dimers to form the senescence antigen on the cell surface,[11] to which IgG binds, signaling red cell removal by the RES. Band 3 clustering implies an atypical lateral mobility within the plane of the cell membrane, ie, one which is unrestricted by the constraints of the spectrin network. We envision that globin-spectrin complex formation and the consequent inhibition of spectrin dimer self-association provides for the loosening of

constraints on band 3 lateral mobility.

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Table 1. Incremental increase in spectrin tetramer concentration as a function of peroxide treatment in vitro

μM H_2O_2	% tetramer increase[a]
0	40 ± 4
147	19 ± 2
489	9 ± 4
CO+489	39 ± 5

[a]incremental increase, average of two trials

Incremental (percentage) increases in tetramer concentration over a 4 hour incubation were quantified from equilibration trials of globin-spectrin complexes produced at 0, 147 and 489 μM H_2O_2 and of carboxyhemoglobin/peroxide treated spectrin, as illustrated for one trial in Fig. 1A, 1D, 1F and 1G.

Table 2. Spectrin extractability from peroxide-treated red cells

μM H_2O_2	% extracted spectrin[a]
0	80 ± 13
349	71 ± 18
849	42 ± 18

[a]relative to total spectrin in corresponding cell ghosts, average of three runs

Equal numbers of cell ghosts and equal volumes of extracted spectrin from each peroxide treatment were electrophoresed.

Table 3. Compartmentalization of h1 and h2 complexes

cell fraction	μg	area units[a]		area units/μg	
		h1	h2	h1	h2
ghost	2.00	41.8	99.3	20.9	44.6
residual	1.40	0	46.9	0	33.5
extr sp	0.60	28.0	55.3	46.7	92.2

[a]integrated densitometric scans of hh-1 probed bands, Fig. 2

Protein sample sizes chosen for residual membranes and extracted spectrin were empirically determined so that μg ghost = μg (residual + extr sp), as well as $h1_{ghost} \approx h1_{extr\ sp}$, and $h2_{ghost} \approx h2_{residual} + h2_{extra\ sp}$.

Table 4. Inextractable spectrin components

component	area units[a]
h1	0
band 1	6.65
h2	(6.29)[b]
band 2	(13.31)[b]

[a]integrated densitometric scan of gold-stained residual ghost profile, Fig. 2

[b]area units calculated from divided chromatogram peak

Fig 1. Spectrin dimer-tetramer equilibration trials. Each stained gel pair displays dimer (D) and tetramer (T) bands both at the start (O) and after the 4 hour equilibration. A, spectrin, 1 mg/ml; B, 6μM Hb + spectrin; C, spectrin + 147 μM H_2O_2; D, Hb + spectrin + 147 μM H_2O_2; E, spectrin + 489 μM H_2O_2; F, Hb + spectrin + 489 μM H_2O_2; G, Hb (pretreated with CO) + spectrin + 489 μM H_2O_2. Ten microliters was loaded per gel.

Fig 2. Split-lane Western blot analysis of red cell membrane proteins for high molecular weight globin complexes. Reduced proteins from unfractionated cell ghosts (2.00 μg), residual membranes after spectrin extraction (1.40 μg), and the extracted spectrin (0.60 μg) were electrophoresed and transferred to nitrocellulose. The blotted lane positions are denoted at the top. Vertical cuts through the center of each lane created four panels, two of which (labeled "hh-1") were probed with the anti-globin monoclonal antibody hh-1, and developed using the alkaline phosphatase:BCIP/NBT system. The other two (unlabeled) panels were stained with colloidal gold to reveal the total protein profile of each lane. Positions of the globin-spectrin complexes (h1 and h2), bands 1, 2, 3 and globin are indicated on the left.

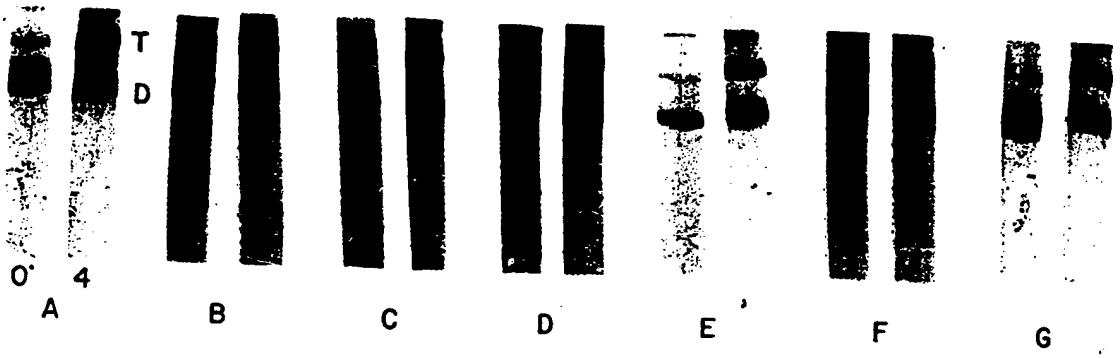

Figure 1

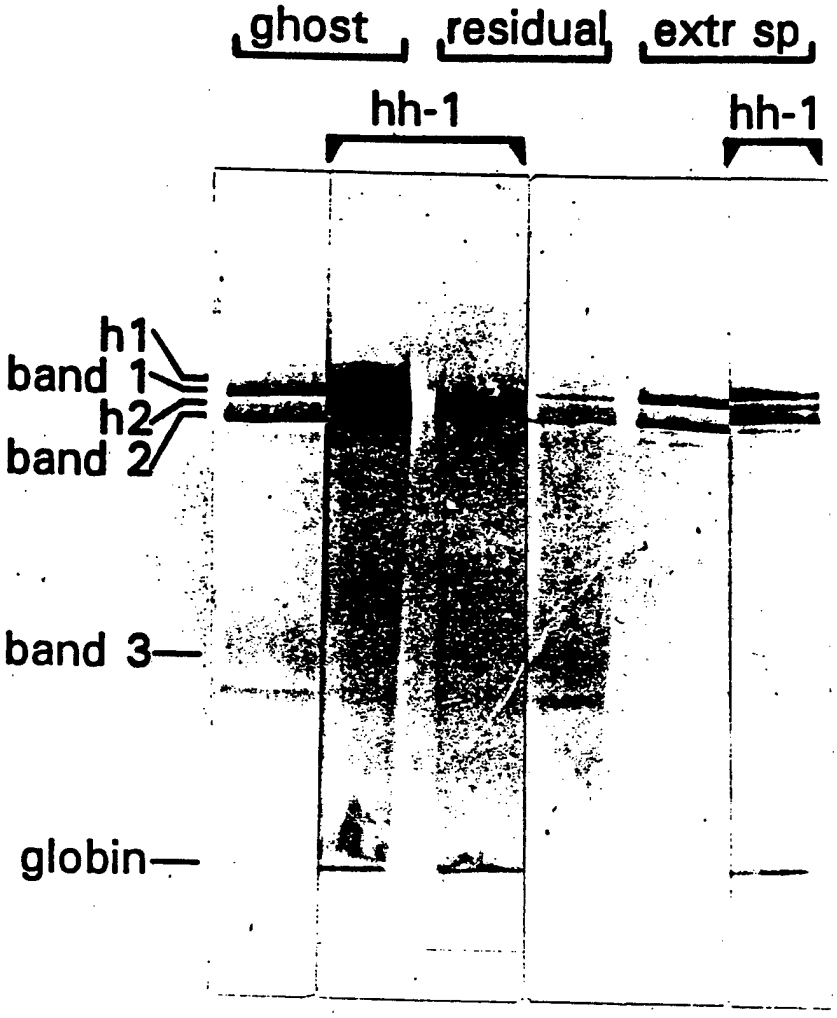
ghost
residual
extr sp
hh-1
hh-1
h1
band 1
h2
band 2
band 3
globin

Figure 2